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Associations between maternal genotypes and metabolites implicated in congenital heart defects

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Abstract

Background—The development of non-syndromic congenital heart defects (CHDs) involves a complex interplay of genetics, metabolism, and lifestyle. Previous studies have implicated maternal single nucleotide polymorphisms (SNPs) and altered metabolism in folate-related pathways as CHD risk factors.

Objective—We sought to discover associations between maternal SNPs and metabolites involved in the homocysteine, folate, and transsulfuration pathways, and determine if these associations differ between CHD cases and controls.

Design—Genetic, metabolic, demographic, and lifestyle information was available for 335 mothers with CHD-affected pregnancies and 263 mothers with unaffected pregnancies. Analysis was conducted on 1160 SNPs, 13 plasma metabolites, and 2 metabolite ratios. A two-stage multiple linear regression was fitted to each combination of SNP and metabolite/ratio.

Results—We identified 4 SNPs in the methionine adenosyltransferase II alpha (*MAT2A*) gene that were associated with methionine levels. Three SNPs in tRNA aspartic acid methyltransferase 1 (*TRDMT1*) gene were associated with total plasma folate levels. Glutamylcysteine (GluCys) levels were associated with multiple SNPs within the glutathione peroxidase 6 (*GPX6*) and O-6-methylguanine-DNA methyltransferase (*MGMT*) genes. The regression model revealed interactions between genotype and case-control status in the association of total plasma folate, total glutathione (GSH), and free GSH, to SNPs within the *MGMT*, 5,10-methenyltetrahydrofolate synthetase (*MTHFS*), and catalase (*CAT*) genes, respectively.

Conclusions—Our study provides further evidence that genetic variation within folate-related pathways accounts for inter-individual variability in key metabolites. We identified specific SNP-

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metabolite relationships that differed in mothers with CHD-affected pregnancies, compared to controls. Our results underscore the importance of multifactorial studies to define maternal CHD risk.

INTRODUCTION

Congenital heart defects (CHDs) are the most prevalent of all structural birth defects, occurring in 8 to 11 of every 1000 live births [1, 2]. Multiple genes have been implicated in CHD development [3], but for the majority of infants diagnosed with a CHD, an established causative gene or teratogenic agent cannot be identified [4, 5]. The majority of CHDs are non-syndromic and are thought to result from a complex interplay between genetic and epigenetic susceptibilities, and parental environmental, lifestyle, and endogenous factors [6]. Due to strong evidence that maternal intake of folic acid supplements reduces the risk of CHDs [6, 7], multiple research studies have focused on the identification of genetic and metabolic risk factors within folate metabolism that are associated with CHDs.

Previous work by our research group and others have described an association between CHDs and alterations in maternal plasma metabolites involved in folate metabolism [8–12]. Studies attempting to identify common genetic variants in genes encoding enzymes involved in folate metabolism associated with an increased risk of CHDs have yielded inconsistent results [13–16]. However, the associations between common genetic variants and plasma metabolites in folate metabolism, and their potential associations with CHDs, remain relatively unexplored.

Nutrigenomics has been defined as the study of how genetic variants influence metabolism and is a rapidly developing field in the study of complex diseases [17]. Compared to strictly genomic studies, combining metabolic and genetic data for nutrigenomic studies (a) provides biochemical measurements which may provide details more directly related to the disease, while (b) genetic variants associated with changes in metabolic homeostasis may provide insight into the pathophysiology of disease [18]. Genome-wide association (GWA) studies have identified and/or verified single nucleotide polymorphisms (SNPs) associated with a few key metabolites involved in folate metabolism [19–22]. However, the majority of large population nutrigenomic studies have not focused on identifying gene-metabolite relationships associated with a particular disease state.

It has become abundantly clear that multifactorial analysis is crucial in determining the risk of complex diseases such as CHDs. We hypothesize that certain maternal common genetic variants are associated with plasma metabolites involved in folate metabolism, and examined the association between 1160 SNPs in 62 genes and 15 selected metabolites and metabolite ratios involved in three key folate-related pathways. We also sought to determine if these associations differed in women who gave birth to infants with a CHD compared to healthy controls.

SUBJECTS AND METHODS

Study Population

All study subjects were enrolled in the National Birth Defects Prevention Study (NBDPS), an ongoing multi-site population-based case-control study to investigate the etiology of 30 non-syndromic birth defects. The NBDPS is the largest case-control study of birth defects ever conducted in the U.S. The study population and eligibility criteria for the NBDPS have been previously outlined [23]. Briefly, subjects were identified through population-based birth defect surveillance systems in 9 states. For the current study, cases are defined as Arkansas mothers who delivered a singleton live birth with a CHD, and participated in a

follow-up study that involved a home visit. Cases in which the pregnancy was also affected by a known single-gene disorder, chromosomal abnormality, or syndrome were excluded. All diagnostic tests, including echocardiograms, cardiac catherizations, surgical and autopsy reports, on Arkansas NBDPS case infants with cardiac defects were reviewed by a pediatric cardiologist to ensure that uniform criteria were used for diagnoses. Using a classification system developed for the NBDPS, which incorporated three dimensions of cardiac phenotype, cardiac complexity, and extracardiac anomalies [24], case women for the current study included those who carried fetuses with at least one cardiac lesion, including conotruncal, septal, and/or obstructive lesions. Controls were those women who had a singleton live birth without birth defects during the same period as cases, who participated in the NBDPS, and were randomly selected from birth certificate data or hospital discharge logs. Case and control mothers spoke either English or Spanish. All study participants submitted buccal cell samples and/or peripheral blood, from which DNA was isolated and plasma metabolites were measured. The study was approved by the University of Arkansas for Medical Sciences' Institutional Review Board and the NBDPS with protocol oversight by the Centers for Disease Control and Prevention (CDC) Center for Birth Defects and Developmental Disabilities. All of the study subjects gave written informed consent, with recruitment commencing in 1998. Finally, women who were pregnant at time of phlebotomy or who had a genotyping call rate <80% were excluded from analysis. The analytical data set used in statistical models contained 335 cases and 263 controls.

Collection of DNA from buccal cell and peripheral blood samples

Methods for NBDPS biologic sample acquisition and processing are well-established [25]. Once a mother has completed the interview, a sample collection kit (containing instructions, consent forms, sterile brushes, reimbursement, and return envelope) is mailed. Blood samples were collected by research nurses during home visits in Arkansas.

Maternal Interview and Home Visit Data

Collection of other covariates has been previously described by our research group [26]. Information regarding selected lifestyle factors were obtained by research nurses using a structured computer-assisted telephone interview and a Block Abbreviated Food-Frequency Questionnaire [27]. After NBDPS participation, each CHD-eligible and control mother participant was contacted by telephone to schedule a home visit. During home visits, the nurse obtained written consent, performed venipuncture to obtain blood, and collected information about current use of multivitamins, cigarettes, alcohol, and caffeine. The selected patient characteristics are listed and summarized in Table 1.

Biochemical measurements

We have previously reported on plasma concentrations of biomarkers in the folate and transsulfuration pathways [8, 9, 26]. Methods for sample preparation and measurement of 14 selected metabolites have been described previously [28, 29]. The metabolites included in the current study are listed and summarized in Table 2. We included all Arkansas NBDPS participants for which metabolite and genetic data had been collected.

Selection of candidate genes and SNPs

Selection of candidate genes was based on the following three inclusion criteria: 1) the gene encodes an enzyme in one of the three candidate metabolic pathways (folate, homocysteine or transsulfuration pathways), as indicated by an advanced search of HumanCyc, a Pathway/Genome Database [30], which is part of the MetaCyc Database Collection [31] and allows human metabolic processes to be viewed within the context of the annotated human genome; 2) the candidate gene is expressed in liver and/or heart tissue [30], and 3) the identity of the

gene is validated by a search of the National Center for Bioinformatics (NCBI) databases [32]. Sixty-two genes that met the above inclusion criteria were chosen and are listed in Table 3.

Haplotype-tagging SNP Selection

Using data from Phase I and Phase II of the International HapMap Project, we have selected a maximally informative set of haplotype-tagging SNPs (htSNPs) for each of the selected 62 candidate genes using an algorithm based on the linkage disequilibrium statistic r^2 [33]. For each gene, htSNPs were chosen from the entire gene region (including introns) with additional 10-kb flanking sequences. To choose htSNPs, pairwise r^2 values were computed for each marker combination within 200 kb for loci with a minor allele frequency (MAF) >0.10 in each population studied. In collaboration with Illumina, htSNPs were chosen based on an Illumina assay design score. SNPs having the highest GoldenGate assay design scores were assigned to haplotype bins using an Illumina proprietary algorithm to predict genotype success. The algorithm provided an overall score that ranges from 0–1 and is based on the predicted optimal oligonucleotide probe sequences for each marker. The oligonucleotide sequences were evaluated for characteristics that include G–C content, melting temperature, self-complementarity, and uniqueness in the genome.

Using these algorithms, a set of htSNPs were selected for inclusion in a customized Illumina GoldenGate genotyping panel. The customized SNP Panel, referred to as an Oligo Pool All (OPA), included a total of 1536 SNPs.

DNA extraction and quantification

DNA was extracted from buccal cell samples and peripheral blood by use of Pure Gene DNA purification reagents (Qiagen, Valencia, CA) according to the manufacturer's protocol, and was quantified by use of the Applied Biosystems (Applied Biosystems, Foster City, CA) TaqMan RNaseP Detection Reagents using a standard curve of DNA of known concentration. Standard curve and DNA samples from case and control subjects were subjected to an initial denaturation at 95°C for 10 min followed by 40 cycles of polymerase chain reaction (PCR) of 95°C for 15 sec and 60°C for 1 min in an ABI PRISM 7900HT real-time PCR instrument. DNA concentrations were calculated from the standard curve using ABI software.

Whole genome amplification

The DNA isolated from buccal cells (10 to 15 ng) was used as a template for whole genome amplification (WGA) by use of the GenomePlex WGA kit according to the protocol provided by the manufacturer (Sigma, St. Louis, MO). The resultant WGA product was quantified as above and 200 ng were used for genotyping in the Illumina Golden Gate assay. The merits of using whole genome amplified DNA in microarray platforms have been previously shown [34].

Genotyping by Illumina Golden Gate Assay

SNP genotyping was conducted using 200 ng (40 ng/μl) of study subject WGA amplified DNA using the Illumina Golden Gate platform [35] and the previously described custom panel of 1536 SNPs in 62 target genes in the folate, homocysteine, and transsulfuration pathways. Genotype analysis was conducted using the Golden Gate assay protocol supplied by Illumina. BeadChips were scanned on the Illumina BeadArray Reader and initial genotype calls were generated using GenCall, Illumina's proprietary genotyping algorithm.

Among the 1536 SNP intensity plots, we observed a varying quality of genotype clustering, with the majority of SNPs displaying three well-segregated genotype clusters, but with a

large number of SNPs clustering poorly; this same behavior was observed in a test set of blood samples. The initial calls, plus the raw intensity data, were therefore used as inputs to SNPMClust, a bivariate Gaussian model-based genotype-clustering and -calling algorithm developed in-house. SNPMClust is an extension of MClust [36], a contributed package in the R statistical computing environment [37], and provides a quantitative measure of genotype-calling uncertainty, which was used to distinguish calls from no-calls. After running SNPMClust on all 1536 SNPs, clustering and classification plots were visually inspected, in some cases leading to dropping a SNP from analysis or applying SNPMClust under non-default settings.

Statistical analysis

SNPs with call rates <90%, SNPs that significantly deviate by race from Hardy– Weinberg equilibrium ($p<10^{-4}$) [38], and SNPs that are monomorphic in our samples were excluded from further analysis. As stated earlier, subjects with call rates <80% of SNPs were also dropped from analysis. This resulted in an analysis data set of 1160 SNPs for 335 case mothers and 263 control mothers.

We initially tested for SNP/metabolite associations assuming three different modes of inheritance (additive, dominant, and recessive), and each of the three models identified different sets of significant SNPs. Lettre et al. [39] have shown that the codominant, or "genotypic", model retains nearly optimal statistical power under all three modes of inheritance, even though it is a general model that makes no assumptions about the contribution of each additional risk allele. By using a single test of association, furthermore, there is no need to adjust for the bias that is introduced by running three models and then choosing the most significant result. All of the association results we present, therefore, were generated under the co-dominant model.

Our previous research [26] has shown significant associations between CHD status and multiple metabolites in the folate pathway. Case-control status was therefore included as a dummy variable in all of the association analysis. We also observed multiple SNP/metabolite associations with non-additive interactions between genotype and disease status; that is, instances where the magnitude, or even direction, of genetic association differs between cases and controls. Simultaneously testing for association and interaction, however, reduces the statistical power to detect associations in the absence of interactions. We therefore implemented a model selection procedure, in which genotype/case-control interaction terms were included in the model only if a likelihood ratio test found these terms to be significant at $p<0.01$. Additional environmental and behavioral variables included in the model were age, BMI, race, interval between birth and phlebotomy, nursing status, smoking status, drinking status, and vitamin-intake status at time of phlebotomy.

To account for the large number of statistical tests being performed, false discovery rate (FDR) q-values [40] were computed for each SNP on a metabolite-bymetabolite basis. In our context, FDR is defined as the proportion of detected SNPs that are, in truth, not associated with the metabolite, while the q-value is defined as the minimum FDR at which a given test can be considered significant. The q-value is thus the FDR equivalent of the p-value and was computed using the *qvalue* package in the R statistical programming environment [37]. We also produced quantile-quantile (Q-Q, WTCCC 2007) plots of $-\log10(p\text{-values})$, which allow visual inspection of the distribution of p-values across all SNPs. Deviations above the dotted line of equality indicate a distribution of p-values more heavily weighted toward zero than expected under the null hypothesis of no genetic association. Statistical analysis was performed in the R statistical programming environment (37).

RESULTS

Patient characteristics

A total of 335 cases (mothers with CHD-affected pregnancies) and 263 control mothers were included in the study. Patient characteristics are summarized in Table 1. Briefly, post-pregnancy (at time of home visit and phlebotomy) alcohol drinking tended to be more prevalent (p=0.064) in cases (71.9%) than in controls (63.5%). A higher proportion (p=0.073) of case mothers tended to be overweight (25.7%) compared to the control population (20.5%), consistent with previously published analysis of NBDPS data [41]. The prevalence of vitamin supplementation and smoking was not significantly different in cases compared to controls.

Plasma metabolite and SNP summary with quality control

The 13 metabolites (and 2 metabolite ratios) with their corresponding means and standard deviations are summarized in Table 2. Plasma metabolites did not display a normal distribution, thus all metabolites were log-transformed prior to analysis. Comparisons between cases and controls agreed with our previously reported findings that alterations in maternal plasma metabolites are associated with CHDs. For genetic analysis, a custom Illumina GoldenGate Panel consisting of 1536 SNPs in 62 genes involved in the homocysteine-homocysteine, folate, and transsulfuration pathways was assayed (Table 3). As described above, 1160 SNPs met our quality control standards and were included in the statistical analysis.

SNP and metabolite associations

The first goal of the present study was to determine associations between the 1160 SNPs and 13 metabolites (and 2 metabolite ratios) involved in three key folate-related pathways for the entire sample population (cases and controls). The top 20 associations for each metabolite are listed in Supplemental Table 1. Quantile-quantile (Q-Q) plots were generated for each metabolite and are displayed in Supplemental Figure 1. The Q-Q plots provide visual evidence that multiple metabolites have significant SNP associations. Figure 1 displays a Manhattan plot for each metabolite, showing the $-\log_{10}$(p-value) vs. the physical location of each SNP, and highlighting associations grouped within specific genes and gene families. The grouping of multiple significant SNPs within genes reflect patterns of linkage disequilibrium (LD) in nearby loci.

Among the top 20 SNP associations for plasma methionine, 2 SNPs (rs933271, rs174675) were present in the catechol-O-methyltransferase (*COMT*) gene, and 4 SNPs (rs2028898, rs762684, rs17026447, rs17026396) resided in the methionine adenosyltransferase II alpha (*MAT2A*) gene. Both genes are involved in the homocysteine pathway. The most significant associations for adenosine levels included 2 SNPs (rs1801394, rs162027) in the methionine synthase reductase (*MTRR*) gene. Both adenosine and the *MTRR* gene are involved in the homocysteine pathway. Homocysteine levels were associated with 2 SNPs (rs9621049, rs4820886) in the transcobalamin II (*TCN2*) gene, and 2 SNPs (rs2042235, rs8177427) in the glutathione peroxidase 3 (*GPX3*) gene. Additionally, 3 of the top 4 associations for total plasma folate levels included SNPs (rs12241572, rs11254397, rs7085709) in the tRNA aspartic acid methyltransferase 1 (*TRDMT1*) gene.

For metabolites in the transsulfuration pathway, multiple associations were also observed. Within the top 20 associations for glutamylcysteine (GluCys) levels, 6 SNPs (rs2859355, rs406113, rs1002259, rs974334, rs4713167, rs2859358) are located in the glutathione peroxidase 6 (*GPX6*) gene, and 6 SNPs (rs9971190, rs9299872, rs7080570, rs11016857, rs7923750, rs7098295) within the O-6-methylguanine-DNA methyltransferase (*MGMT*)

gene. GluCys and the *GPX6* gene are both involved in antioxidant processes in the transsulfuration pathway. Free GSH levels were associated with 5 SNPs in the glutathione S-transferase alpha (*GSTA*) family of enzymes. The associations included 3 SNPs in the *GSTA3* gene (rs641019, rs1052661, rs9463842), 1 SNP (rs6902065) in the *GSTA2* gene, and 1 SNP (rs654144) in the *GSTA4* gene. For the ratio of reduced and oxidized glutathione (GSH/GSSG), a measure of cellular redox potential, 5 SNPs (rs2975138, rs2975139, rs7294985, rs6488840, rs11056890) in the microsomal glutathione S-transferase 1 (*MGST1*) gene were included among the top 20 associations. Taken together, the described results lend further evidence that SNPs located in genes involved in folate metabolism are associated with plasma metabolites in the homocysteine, folate, and transsulfuration pathways.

Among all metabolites, only two SNPs achieved an FDR q-value < 0.05, namely, two adjacent SNPs (rs3797546, rs16876528) in the betaine-homocysteine methyltransferase (*BHMT*) gene that were significantly associated with reduced total glutathione (GSH). Further inspection revealed that these associations were primarily due to a single patient with the lowest observed GSH in our sample and the homozygous minor allele genotype at both SNPs; we therefore treat this result with reservations for the time being.

Investigation of case-control and genotype interactions

One goal of this study was to determine if the relationship between specific SNPs and metabolites differed between cases and controls. As described in the Statistical Analysis section of Methods, each combination of metabolite and SNP was tested for a non-additive interaction between genotype and case/control status, and plots of three illustrative findings are shown in Figure 2. In Figure 2a, higher folate levels were observed among controls for the homozygous minor genotype (GG) at SNP rs10764896, located in the *MGMT* gene, than for the two other genotypes. The opposite trend was observed in cases (p=0.0012, see Supplemental Table 1), as the homozygous GG genotype had lower folate levels. In Figure 2b, free GSH levels in controls with the minor AA genotype at SNP rs2733103, located within the methenyltetrahydrofolate synthetase (*MTHFS*) gene, were lower than both other genotypes. The same pattern was observed among cases, but with a greater difference (p=0.0061). Finally, in Figure 2c, controls possessing the homozygous minor TT genotype at SNP rs564250, in the catalase (*CAT*) gene, had lower total GSH levels compared to the other two genotypes, while controls show the opposite pattern (p=0.0008). These and several other interactions between case-control status and genotype (Supplemental Table 1) provide evidence that multiple SNP-metabolite associations do indeed differ depending on CHD status.

DISCUSSION

Our study builds on previous ones that have shown that maternal SNPs and metabolites involved in folate metabolism are associated with CHDs. In a recent study, we showed that elevated homocysteine, decreased methionine, and reduced GSH/GSSG ratio were significant factors in discriminating between CHD case and control mothers [26]. The goal of the current study is to 1) investigate the relationship between 1160 maternal SNPs, 13 metabolites, and 2 metabolite ratios involved in folate metabolism and 2) determine if any associations between maternal SNPs and metabolites differ between cases and controls. The study represents a comprehensive investigation combining both genetic and metabolic data in CHDs. Our study differs from previous ones, in that we not only investigated gene-metabolite associations in our population, but determined if particular SNP-metabolite associations differed in mothers with CHD-affected pregnancies compared with controls.

The associations observed in our study include genes and metabolites in three key interconnected pathways involved in folate metabolism. Multiple SNPs in *COMT* and *MAT2A* were found to be associated with methionine levels. Methionine is an essential amino acid, crucial in synthesis of methyl groups for methyltransferase reactions. *COMT* is involved in vascular and metabolic homeostasis and has been shown to interact with methylenetetrahydrofolate reductase (*MTHFR*), a key enzyme in folate metabolism [42]. It is postulated that the association between *MTHFR* and *COMT* may exist because *MTHFR* modulates the availability of methyl groups, which are the cosubstrate for *COMT* reactions. *MAT*s are involved in the synthesis of SAM from methionine [43], and SNPs within the *MAT*s have been implicated in hypertension [44]. The top 2 SNPs (rs9621049, rs4820886) associated with homocysteine levels were found within the *TCN2* gene. The rs9621049 SNP results in an amino acid change from serine to phenylalanine (S348F), which has not yet been correlated with enzyme activity. Both rs9621049 and rs4820886 were investigated in a recent study that implicated TCN2 genetic variation in homocysteine levels, but neither SNP was found to be significantly associated [45]. Elevated homocysteine, a key metabolite in folate metabolism, has been associated with a variety of disease states including cardiovascular disease, Alzheimer's disease, osteoporosis, and birth defects including CHDs [46]. Previous studies have discovered that genetic variation within the *TCN2* gene is associated with plasma homocysteine concentrations [47]. We found that multiple SNPs within *TRDMT1* were associated with plasma folate levels. *TRDMT1* codes for a methyltransferase that methylates the aspartic acid transfer RNA [48]. A previously reported study observed an association between genetic variation within *TRDMT1* and spina bifida, and found the same variant was associated with red blood cell folate levels [49].

Multiple associations at SNPs in the *GPX6* and *MGMT* genes were observed for glutamylcysteine (GluCys). Both genes are thought to be involved in the prevention of DNA damage and oxidative stress, and GluCys represents a key intermediate in the conversion of cysteine to glutathione, an important cellular antioxidant metabolite. Six SNPs in *GPX6* and *MGMT* were associated with GluCys levels. The *GPX* family of genes is instrumental in the scavenging of reactive oxygen species (ROS) that could arise from oxidative insults, and genetic variation within *GPX6* may alter glutathione metabolism. SNPs in the *GPX* gene family have been shown to alter enzyme activity and increase the risk of thrombotic events [50]. Although the direct link between SNPs in the *MGMT* gene and GluCys remains unclear, previous studies have investigated the relationship between antioxidant mechanisms and *MGMT* gene expression [51].

Free glutathione levels (GSH) are essential in antioxidant mechanisms [52]. Five SNPs in the *GSTA* gene family were associated with free GSH concentrations. *GSTA* is involved in detoxification mechanisms through conjugation with GSH [53]. Thus, SNPs within *GSTA* genes may influence glutathione metabolism.

We investigated whether the relationship between specific SNPs and metabolites differed between cases and controls. Due to the complexity of cardiogenesis, it is plausible that both genetic and metabolic insults may be involved in CHD development. As shown in Figure 2, lower total plasma folate and lower free GSH were observed for the homozygous minor genotype for SNPs in *MGMT* (rs10764896) and *MTHFS* (rs2733103), respectively, in cases. The direction of the metabolic alterations in case mothers are in agreement with our research group's previous findings of perturbations in maternal folate metabolism being associated with CHDs [8, 9, 26]. The association of lower free GSH in case mothers possessing the minor genotype for *MTHFS* (rs2733103) is in agreement with the hypothesis that alterations in methionine metabolism may lead to decreased cycle turnover, and may ultimately lead to decreased glutathione synthesis [52]. *MTHFS* is a key enzyme involved in the folate pathway that metabolizes formyltetrahydorolate. The folate pathway is

interconnected with methionine metabolism by providing the methyl groups for the remethylation of homocysteine back to methionine. SNPs within *MTHFS*, furthermore, have been associated with non-syndromic cleft lip and palate [54].

A few limitations should be noted. First, maternal blood was drawn after pregnancy, and metabolite levels measured in the study may not be reflective of metabolism during cardiogenesis. Multiple studies have shown, however, that homocysteine concentrations and dietary patterns remain relatively stable from preconception throughout pregnancy and postpartum [55, 56]. Second, we investigated only common genetic variants in folate metabolism. Other associations might be observed with the inclusion of rare variants, additional genes in folate metabolism, and other metabolites related to folate metabolism. Third, our study included only mothers and not offspring. Identification of genetic risk factors is complicated by the fact that both maternal and fetal genetic susceptibilities may affect the intrauterine environment during gestation and contribute to the development of CHDs [57, 58]. Nevertheless, assessing maternal factors in CHDs is crucial, as the developing fetus requires the maintenance of homeostasis in the maternal environment. Maternal genetic associations, furthermore, may aid in assessing preconception risk and be amenable to targeted clinical intervention. An additional limitation of the study is that our case study population consists of a heterogeneous class of CHDs. It is possible that certain genemetabolite interactions may be specific to particular CHD phenotypes (i.e. conotruncal, obstructive, septal) and were not found in this study. Finally, only two SNP/metabolite associations achieved an FDR q-value < 0.05. Although patterns observed in the Manhattan and Q-Q plots (Figure 1 and Supplemental Figure 1) give strong evidence of multiple, genuine associations, the relatively modest sample size prevented this study from reaching the most stringent standards of multiplicity-adjusted Type I error control. Independent replication of our findings will be important to verify the associations identified in our study.

Our study nevertheless represents the most comprehensive investigation to date on the role of maternal SNPs and metabolites in the occurrence of CHDs, and future studies that consider the complex pathophysiology of the formation of CHDs may lead to the discovery of novel risk factors. The discovery of associations between genes and metabolites involved in folate metabolism provides insight into genetic and metabolic profiles associated with CHDs, and may implicate potential mechanisms involved in CHD development. Expansion of nutrigenomic studies in CHDs is warranted, as it becomes clear that a combination of multiple risk factors is involved in the development of non-syndromic CHDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BHMT	betaine-homocysteine methyltransferase gene
CAT	catalase gene
CHDs	congenital heart defects
COMT	catechol-O-methyltransferase gene
FDR	false discovery rate
GluCys	glutamylcysteine
GPX3	glutathione peroxidase 3 gene
GPX6	glutathione peroxidase 6 gene
GSH	glutathione
GSTA	glutathione S-transferase alpha
GSTA2	glutathione S-transferase alpha 2 gene
GSTA3	glutathione S-transferase alpha 3 gene
GSTA4	glutathione S-transferase alpha 4 gene
GWA	genome-wide association
htSNPs	haplotype-tagging SNPs
MAF	minor allele frequency
MAT2A	methionine adenosyltransferase II alpha
MGMT	O-6-methylguanine-DNA methyltransferase gene
MGST	microsomal glutathione S-transferase 1 gene
MTHFRI	methylenetetrahydrofolate reductase
MTHFS	5,10-methenyltetrahydrofolate synthetase gene
MTRR	methionine synthase reductase gene
NBDPS	National Birth Defects Prevention Study
PCR	polymerase chain reaction
ROS	reactive oxygen species
SAM	s-adenosylmethionine
SNPs	single nucleotide polymorphisms
TCN2	transcobalamin II gene
TRDMT1	tRNA aspartic acid methyltransferase 1 gene
WGA	whole genome amplification

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Highlights

- We investigate maternal genetic variation and metabolism in congenital heart defects.
- Maternal SNPs and metabolites involved in folate metabolism were analyzed.
- Multiple associations were found between maternal SNPs and metabolites.
- Interactions between SNPs and metabolites were dependent on case-control status.
- Results underscore the importance of multifactorial studies to define CHD risk.

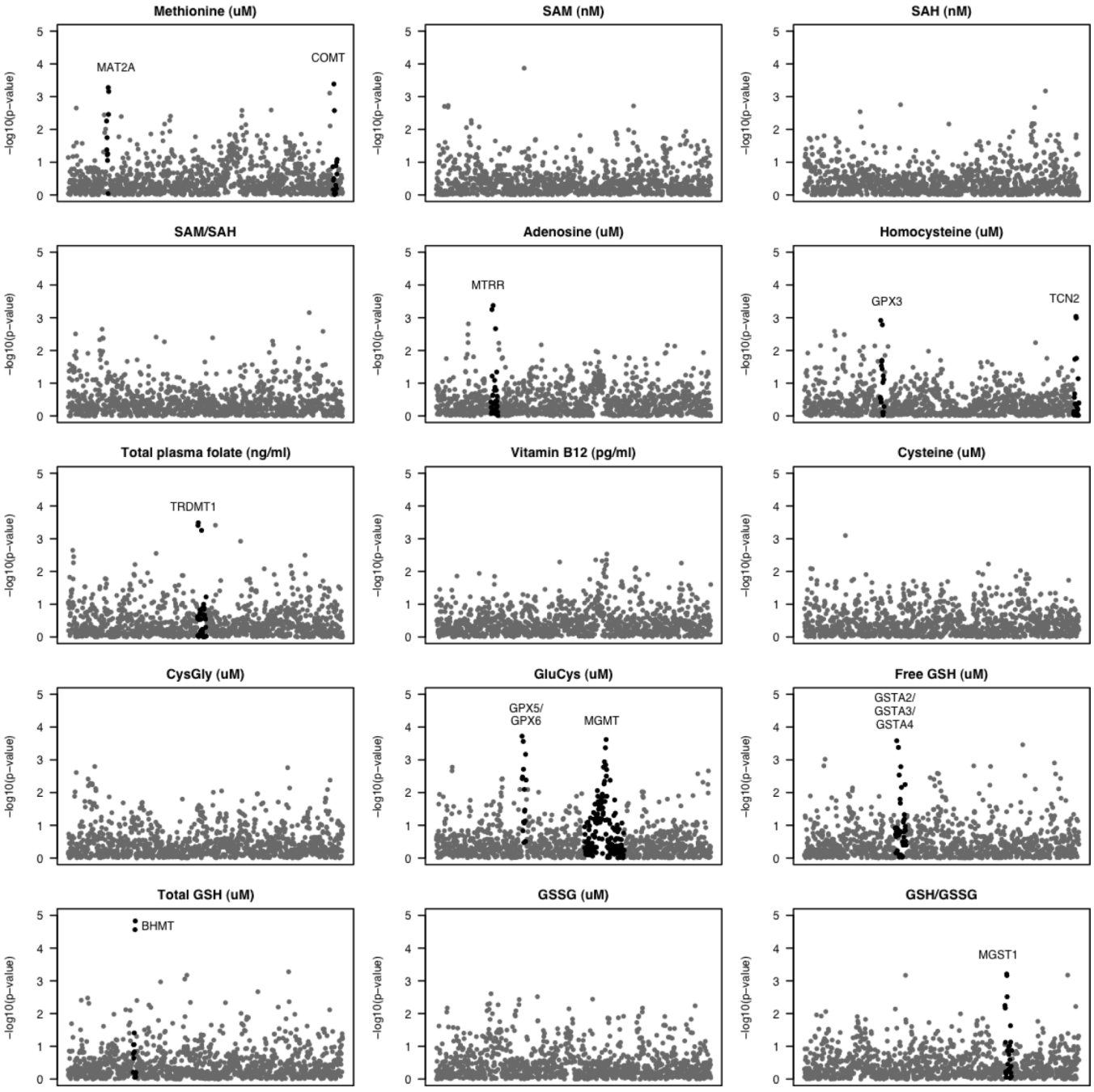

Figure 1. Manhattan plots

Each Manhattan plot shows the $-\log_{10}$(p-value) resulting from a two-stage test of genetic association between each of 1160 SNPs and a given metabolite or metabolite ratio, as described in the text. SNPs are plotted in order of physical location within the genome, and gene-specific associations of interest are highlighted.

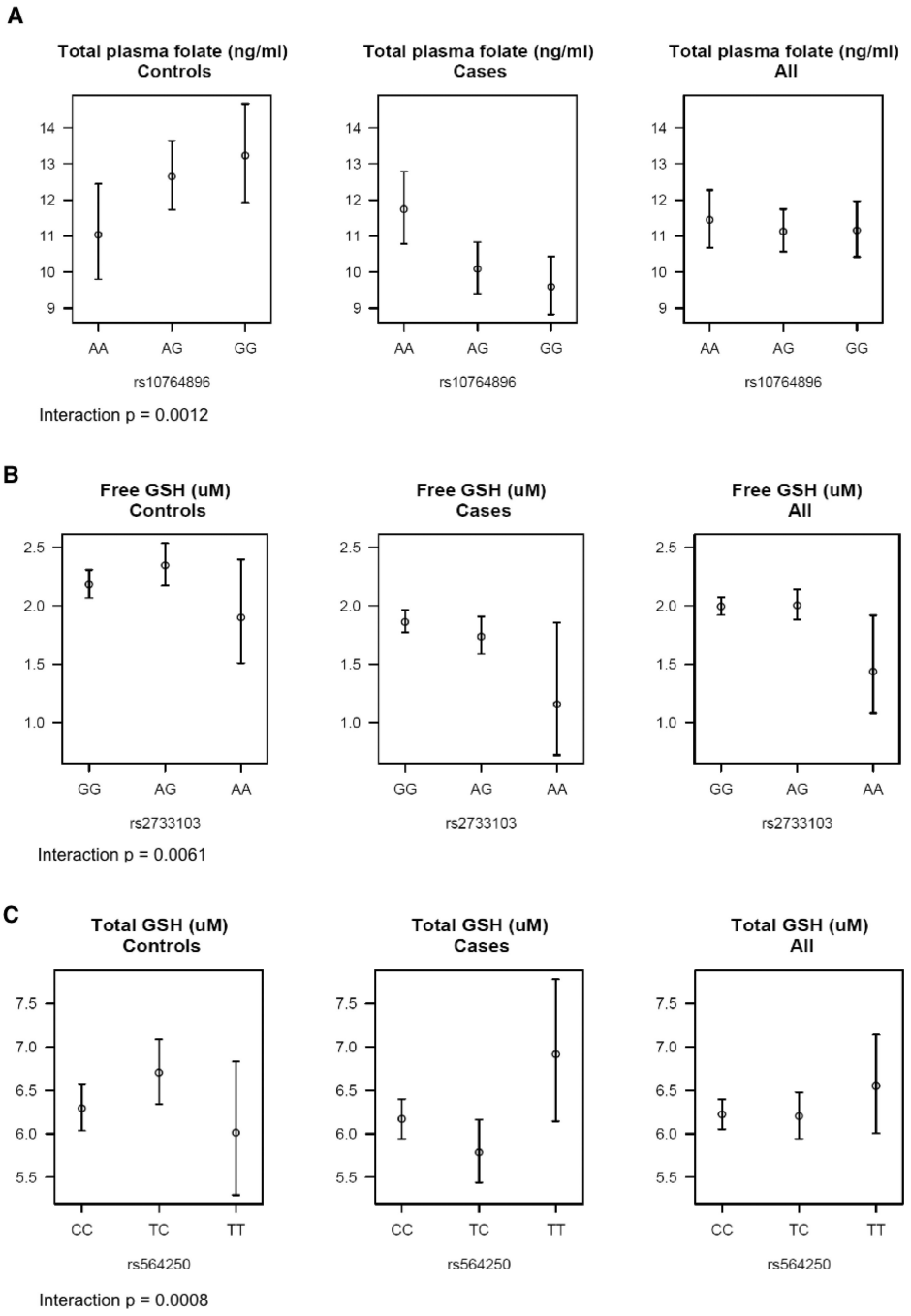

Figure 2. Three associations with genotype/case-control interactions

Mean (+/− 95% CI) metabolite levels, stratified by case-control status and genotype, are given for (2A) total plasma folate and rs10764896 (*MGMT*), (2B) free GSH and rs2733103 (*MTHFS*), and (2C) total GSH and rs564250 (*CAT*). Each association contains a statistically significant interaction ($p<0.01$) between genotype and case-control status, as determined by a likelihood ratio test.

Table 1

Subject Characteristics

Characteristics of 335 case mothers and 263 control mothers, with p-values computed using Fisher's exact test of independence of rows and columns.

	Cases	Controls	
	N (%)	N (%)	p-value
Age (yrs)			
<30	206 (61.5)	160 (60.8)	
>=30	128 (38.2)	103 (39.2)	
Missing	1 (0.3)	0 (0.0)	0.925
Race			
Caucasian	257 (76.7)	199 (75.7)	
African America	49 (14.6)	36 (13.7)	
Hispanic	24 (7.2)	23 (8.7)	
Others	4 (1.2)	5 (1.9)	
Missing	1 (0.3)	0 (0.0)	0.833
Smoker			
No	252 (75.2)	208 (79.1)	
Yes	81 (24.2)	53 (20.2)	
Missing	2 (0.6)	2 (0.8)	0.497
Alcohol drinker			
No	89 (26.6)	88 (33.5)	
Yes	241 (71.9)	167 (63.5)	
Missing	5 (1.5)	8 (3.0)	0.064
Vitamin supplementation			
No	230 (68.7)	177 (67.3)	
Yes	102 (30.4)	83 (31.6)	
Missing	3 (0.9)	3 (1.1)	0.937
BMI status			
Underweight	5 (1.5)	9 (3.4)	
Normal	118 (35.2)	106 (40.3)	
Overweight	86 (25.7)	54 (20.5)	
Obese	115 (34.3)	78 (29.7)	
Missing	11 (3.3)	16 (6.1)	0.073
Maternal Education			
College education or higher	160 (47.8)	140 (53.2)	
High school or less	162 (48.4)	108 (41.1)	
Missing	13 (3.9)	15 (5.7)	0.157
Household Income			
Less than $10,000	58 (17.3)	39 (14.8)	

	Cases	Controls	
	N (%)	**N (%)**	**p-value**
$10,000 – $30,000	116 (34.6)	67 (25.5)	
$30,000 – $50,000	73 (21.8)	64 (24.3)	
More than $50,000	59 (17.6)	57 (21.7)	
Missing	29 (8.7)	36 (13.7)	0.046

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Table 2

Metabolite Summaries

Summary of 13 plasma metabolites and 2 metabolite ratios in 335 case mothers and 263 control mothers, with p-values computed using Welch two sample t-test.

	Cases		Controls		
	N	Mean (Std Dev)	N	Mean (Std Dev)	p-value
Methionine (uM)	335	22.07 (5.21)	263	25.00 (4.58)	<0.0001
SAM (nM)	335	75.13 (16.04)	263	79.11 (14.86)	0.0018
SAH (nM)	335	29.71 (11.68)	263	23.63 (7.60)	<0.0001
SAM/SAH	335	2.89 (1.27)	263	3.67 (1.30)	<0.0001
Adenosine (uM)	331	0.29 (0.17)	257	0.24 (0.13)	<0.0001
Homocysteine (uM)	335	9.20 (2.50)	263	7.46 (1.62)	<0.0001
Total plasma folate (ng/ml)	335	11.40 (4.79)	263	13.66 (6.15)	<0.0001
Vitamin B12 (pg/ml)	335	466.05 (214.99)	263	487.32 (223.15)	0.2401
Cysteine (uM)	335	231.75 (27.13)	263	229.36 (26.38)	0.2773
CysGly (uM)	335	44.24 (7.74)	263	44.01 (7.67)	0.7205
GluCys (uM)	335	2.60 (0.88)	263	2.80 (1.01)	0.0135
Free GSH (uM)	335	1.98 (1.08)	263	2.39 (1.15)	<0.0001
Total GSH (uM)	335	6.33 (1.74)	263	6.64 (1.70)	0.0300
GSSG (uM)	335	0.34 (0.19)	263	0.23 (0.08)	<0.0001
GSH/GSSG	335	22.27 (10.66)	263	31.55 (12.65)	<0.0001

Table 3

Custom SNP Panel

Summary of custom Illumina GoldenGate genotyping panel consisting of 1536 SNPs in 62 genes involved in the homocysteine, folate, and transsulfuration pathways.

Gene Symbol	Chromosome	Number of SNPs	Pathway
MTHFR	1	25	Folate
TYMS	18	22	Folate
TCN2	22	30	Folate
RFC1	4	32	Folate
SHMT1	17	31	Folate
MTHFD1	14	26	Folate
MTHFD2	2	18	Folate
MTHFS	15	45	Folate
DHFR	5	8	Folate
FOLR1	11	7	Folate
FOLR2	11	5	Folate
AHCYL1	1	17	Homocysteine
MTR	1	51	Homocysteine
CTH	1	30	Homocysteine
DNMT1	19	10	Homocysteine
TRDMT1	10	47	Homocysteine
DNMT3A	2	40	Homocysteine
DNMT3B	20	41	Homocysteine
DNMT3L	21	13	Homocysteine
MAT1A	10	32	Homocysteine
MAT2A	2	13	Homocysteine
MAT2B	5	28	Homocysteine
BHMT	5	19	Homocysteine
BHMT2	5	13	Homocysteine
KIAA0828	7	38	Homocysteine
COMT	22	39	Homocysteine
MTRR	5	37	Homocysteine
GNMT	6	7	Homocysteine
CBS	21	38	Transsulfuration
GSR	8	29	Transsulfuration
GCLM	1	10	Transsulfuration
GSS	20	18	Transsulfuration
PGDS	4	21	Transsulfuration
GCLC	6	60	Transsulfuration
MGST1	12	36	Transsulfuration

Gene Symbol	Chromosome	Number of SNPs	Pathway
OGG1	3	8	Transsulfuration
GPX2	14	9	Transsulfuration
GPX3	5	29	Transsulfuration
GPX4	19	7	Transsulfuration
GPX5	6	12	Transsulfuration
GPX6	6	9	Transsulfuration
GLRX	5	32	Transsulfuration
GSTK1	7	2	Transsulfuration
NOS2A	17	35	Transsulfuration
NOS3	7	17	Transsulfuration
GSTA1	6	9	Transsulfuration
GSTA2	6	17	Transsulfuration
GSTA3	6	16	Transsulfuration
GSTA4	6	19	Transsulfuration
SOD1	21	12	Transsulfuration
SOD2	6	11	Transsulfuration
SOD3	4	12	Transsulfuration
CAT	11	41	Transsulfuration
GSTZ1	14	21	Transsulfuration
GSTM2	1	6	Transsulfuration
GSTM3	1	5	Transsulfuration
GSTM4	1	12	Transsulfuration
GSTM5	1	11	Transsulfuration
GSTO1	10	18	Transsulfuration
GSTP1	10	13	Transsulfuration
GSTT2	22	2	Transsulfuration
MGMT	10	215	Transsulfuration
Total		**1536**	